Protocol

Immunometric Antibody Sandwich Enzyme-Linked Immunosorbent Assay

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The antibody sandwich enzyme-linked immunosorbent assay (ELISA) is the most commonly used assay for rapid and accurate detection of antigens. It displays greater sensitivity compared with the indirect ELISA and can be used to determine absolute antigen concentrations in unknown samples provided purified antigen standards are available, although it requires the use of two different antibodies. Briefly, wells are coated with antigen-specific capture antibody then incubated with samples containing unknown antigen. Washing removes unbound antigen and exogenous sample protein before incubation with a second antigen-specific detection antibody, washing, and reincubation with a reporter-labeled tertiary antibody. After tertiary antibody is washed off, substrate is added and hydrolysis is measured spectrophotometrically. The signal intensity is directly proportional to the concentration of the antigen in the test sample.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

3,3',5,5'-tetramethylbenzidine (TMB) (Rockland Immunochemicals, #TMBE-1000) (for horseradish peroxidase [HRP]-based assays)

Antibodies of known concentration for antigen-specific capture and detection

These can be polyclonal/monoclonal, monoclonal/monoclonal, or monoclonal/polyclonal antibody combinations (Rabin et al. 1992; Hornbeck et al. 2001). Addition of a reporter-labeled detection antibody (which eliminates the need for a tertiary antibody) converts this assay into a direct sandwich ELISA (Fig. 1).

Antibody, enzyme-labeled antihost, and dilution buffer (e.g., Rockland Immunochemicals #MB-061-100 or #MB-076-0100)

Antigen at an unknown concentration

Blocking buffer (3% [w/v] fish gel [Sigma-Aldrich, #G7765] in PBS or TBS)

Carbonate buffer for immunoassays <R> (optional; see Step 1)

Detection-antibody diluent (1% [v/v] fish gel in PBS or TBS)

para-Nitrophenyl phosphate (pNPP) (Rockland Immunochemicals, #NPP-10) (for alkaline phosphatase [AP]-based assays)

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Immunometric Antibody Sandwich ELISA

FIGURE 1. Direct sandwich ELISA for the chemiluminescent detection of soluble antigen.

Phosphate-buffered saline (PBS) for immunoassays (pH 7.4) <R>

Stop solution (1 M HCl) (for HRP-based assays only; see Step 14)

Tris-buffered saline (TBS) for immunoassays <R> (optional; see Step 1)

Wash buffer with detergent (PBS or TBS containing 0.05% [v/v] Tween 20)

Equipment

Microplate reader (Bio-Rad, Model #680) with Microplate Manager Software (5.2.1) or equivalent

Microplate sealing tape, polyester, nonsterile (Corning, #4612 or equivalent)

Microtiter plates (flat bottom, 96-well, 0.3 mL per well) with appropriate surface chemistry (e.g., Immulon 4 HBX; Thermo Scientific, #3855 or equivalent)

Multichannel pipettes (eight- or 12-channel) (Costar #4888 or equivalent)

Plate washer (Dynex Ultrawash PLUS or equivalent)

Reagent reservoirs (VWR, #12577-027 or equivalent)

METHOD

1. Dilute the capture antibody to a final concentration of 1 µg/mL in PBS, TBS, or a carbonate buffer. Coat a microtiter plate by pipetting 100 µL of diluted antibody into individual wells.

*For HRP conjugates, use PBS or carbonate buffer. For AP conjugates, use TBS. Dilution of the monoclonal or polyclonal capture antibody to a final concentration of 1 µg/mL serves as an indicator only. It is best to titrate the antibody against the standard antigen by indirect ELISA using the checkerboard titration format (see Protocol: **Indirect Immunometric ELISA** [Kohl and Ascoli 2017]). The final antibody working concentration should be adjusted to the lowest concentration that results in maximum signal strength.*

2. Cover the plate with a lid or plastic adhesive. Incubate for 1 h at room temperature or overnight at 2°C–8°C.
3. Using a plate washer, aspirate the coating solution. Wash the plate three times by filling each well with 300 µL of wash buffer containing detergent. Remove retained liquid by patting the plate on a paper towel.
4. Add 200 µL of 3% (v/v) fish gel in PBS or TBS blocking buffer to block the remaining protein-binding sites. Incubate for 1 h at room temperature or 30 min at 37°C.
5. Aspirate the blocking solution using the plate washer. Wash the plate as described in Step 3.

TABLE 1. ELISA troubleshooting guide

Problem	Possible cause	Recommendation
High background	Insufficient washing	Ensure proper performance of the plate washer for complete removal of residual liquid following the washing steps. Increase the number of washes or lengthen the soaking time between washes.
	Insufficient blocking	Increase blocking time or apply different blocking buffer. Fish gelatin can be stored for up to 2 wk at 2°C–8°C and should be discarded thereafter.
	Secondary antibody dilution too low; increased presence of reporter	Increase antibody dilution, titrate antibody if required.
No signal observed	Incorrect preparation or addition of reagents	Review protocol and repeat the assay. Ensure proper calculation of all dilutions and prepare fresh assay-associated reagents.
	Insufficient antibody applied	Increase antibody concentrations.
	Insufficient antigen presence and binding	Select an alternative microtiter plate displaying different binding characteristics. Dilute antigen in PBS devoid of additional proteins. Increase the antigen amount or change to a more sensitive assay.
	Contaminated buffers	Prepare fresh buffers.
	Contaminated enzyme-labeled secondary antibody	Apply fresh reagents.
	Expired substrate	Prepare fresh substrate.
Excessive signal observed	Insufficient incubation temperature and time	Ensure that all assay-associated steps are performed at the recommended temperatures.
	Insufficient washing; unbound enzyme remaining	Ensure proper performance of the plate washer for complete removal of residual liquid following the washing steps. Increase the number of washes or lengthen the soaking time between washes.
	Insufficient blocking	Increase the blocking time or apply a different blocking buffer.
	Cross-well contamination	Ensure correct use of pipette tips for reagent addition and appropriate application of unused disposable plate sealers.
	Reporter antibody dilution too low; increased presence of reporter	Increase the antibody dilution; titrate the antibody if required. Reduce the incubation time.
	Reporter contamination as a result of reused consumable materials	Use disposable reagent reservoirs and plate sealers. Do not reuse them.
	Contaminated buffers	Prepare fresh buffers.
	Reaction was not stopped as recommended.	Stop reaction as directed.
	Substrate development occurred in the light.	Perform substrate development in the dark.
Plate edge effect	Incubation temperature too high	Ensure that all assay-associated steps are performed at the recommended temperatures.
	Environmental temperature fluctuations	Perform assay within a temperature-controlled environment. Seal the plates completely.
	Contaminated assay-specific reagents	Prepare fresh reagents and buffers.
Positive result in negative control	Cross-reactive antibodies (sandwich ELISA)	Ensure no cross-reactivity between antibodies used in the ELISA.
	Plates stacked during incubations	Avoid stacking plates.
	Pipetting inconsistencies	Ensure careful pipetting using calibrated micropipettes.
Plate absorbance inconsistencies	Excessive drying	Perform assay within a humidity-controlled environment.
	Insufficient washing	Ensure proper performance of the plate washer for complete removal of residual liquid following the washing steps. Increase the number of washes or lengthen the soaking time between washes.
	Dirty plate bottom	Clean the bottom of the plate to allow for accurate absorbance readings.
Poor interassay reproducibility	Insufficient washing	Ensure proper performance of the plate washer for complete removal of residual liquid following the washing steps. Increase the number of washes or lengthen the soaking time between washes.
	Insufficient incubation temperature	Ensure that all assay-associated steps are performed at the recommended temperatures.
	Protocol variations	Ensure adherence to the same protocol for all assays performed.
Slow color development	Contaminated buffers	Prepare fresh buffers.
	Incorrect incubation temperature	Ensure that all assay-associated steps are performed at the recommended temperatures.
	Reporter antibody concentration too low	Decrease antibody dilution; titrate antibody if required.
	Contaminated assay-specific reagents	Prepare fresh reagents and buffers.

6. Prepare a standard antigen dilution at a recommended concentration of 1 µg/mL in PBS. Add 100 µL to selected wells in Column 1. Titrate twofold or another serial dilution across the length of the plate. Incubate for 1 h at room temperature. Antigen blanks and assay blanks do not receive any antigen solution.

For an accurate determination of antigen concentration in unknown samples, the standard antigen dilution series needs to cover the dynamic range of the assay. Defined as the range in which the sensitivity of the immunoassay is determined by the interplay between antigen concentration and signal strength, a derived standard curve permits accurate determination of antigen concentrations within unknown samples. The dynamic range of an assay is antigen dependent and typically spans the femtogram-to-microgram detection range.

7. Add 100 µL of test antigen to selected wells in Column 1. Titrate the test antigen by two-fold or another serial dilution across the length of the plate. Incubate for 1 h at room temperature.

Given the unknown antigen content of test samples, it is best to assay two different, preferentially overlapping, serial dilutions of the test antigen to ensure that a dilution series falls within the assay's dynamic range and allows for accurate measurement. Assay performance over several plates needs to include the standard antigen dilution series as well as assay controls on every plate. Individual samples should be run in duplicate or triplicate for accurate quantification.

8. Aspirate the antigen solution using the plate washer. Wash the plate as described in Step 3.
9. Dilute the detection antibody to a final concentration of 1 µg/mL in 1% (v/v) fish gel in PBS. Pipette 100 µL of the detection antibody into each well across the plate, including the antigen blank control wells. Incubate the plates for 1 h at room temperature.

*Dilution of the monoclonal or polyclonal detection antibody to a final concentration of 1 µg/mL serves as an indicator only. It is best to titrate the antibody against the standard antigen by indirect ELISA using the checkerboard titration format (see Fig. 1 of Protocol: **Indirect Immunometric ELISA** [Kohl and Ascoli 2017]). The final antibody working concentration should be adjusted to the lowest concentration that results in maximum signal strength.*

10. Aspirate the detection antibody solution using the plate washer as described in Step 3.
11. Prepare enzyme-labeled antihost antibody at a recommended dilution of 1:20,000 using the dilution buffer provided with the antibody. Add 100 µL to each well including the assay control wells. Incubate for 30 min at 37°C.

A 1:20,000 dilution of the reporter-labeled antibody is a recommendation only and is dependent on the concentration of the reporter antibody. It is best to titrate the antibody by indirect ELISA using the checkerboard titration format to determine the optimal working concentration. When using self-type antibodies (mouse-derived monoclonal/monoclonal or rabbit-derived polyclonal/polyclonal) in the sandwich ELISA, the detection antibody must be biotinylated or directly labeled to prevent nonspecific cross-reactivity of the reporter-labeled antibody with both the capture and detection antibodies. It is best to use reporter-labeled antibodies in a sandwich ELISA in which the detection and capture antibodies are derived from different species. This guarantees that nonspecific cross-reactivity will not be observed on addition of the tertiary antibody.

12. Using the plate washer, aspirate the reporter-labeled tertiary antibody solution. Wash the plate as described in Step 3.
13. Prepare or equilibrate the substrate solution (TMB for HRP or pNPP for AP) to room temperature. Add 100 µL to each well. Incubate the enzyme-based reactions for 30 min at room temperature in the dark.

Avoid introducing air bubbles when adding substrate to the wells, because these will affect the absorbance readings.

14. Stop the HRP reaction by adding 100 µL of 1 M HCl per well. Alkaline phosphatase-based substrate development does not require a stopping reagent.

For further quantification of low-level reactions, skip Step 14 and perform plate measurements at set time intervals.

15. Read the plates on a microplate reader set to 405 nm for AP-based and 450 nm for HRP-based substrate development.

16. Prepare a standard curve from the data by plotting standard antigen concentration on the x -axis versus absorbance on the y -axis. Use this standard curve to determine the antigen concentration of unknown test samples.

The results from ELISAs using chemiluminescent or fluorescent reporters will be in relative light units (RLUs) instead of absorbance in the standard curve. Standard curves are only required to determine the amount of antigen in test samples accurately; they are redundant for qualitative answers.

Please see Table 1 for a detailed troubleshooting guide.

DISCUSSION

The sensitivity of the sandwich ELISA depends on: (1) the number of molecules of capture antibody immobilized on the well's surface; (2) the avidity of the capture antibody for the antigen; (3) the avidity of the detection antibody for the antigen; and (4) the specific activity of the detection antibody in the ELISA. When using monoclonal/monoclonal or polyclonal/polyclonal combinations, a subset of the antibody must be biotinylated or directly labeled with a reporter of choice to detect the antigen. Furthermore, when using two monoclonal antibodies, they must recognize discrete epitopes on the antigen under investigation.

Optimizing Pairwise Antibody Concentrations

In addition to being suitable for the detection or quantification of different antigens and antibodies, the sandwich ELISA is essential for identifying suitable antibody pairs for assay development. Here we provide an example that uses the sandwich ELISA to determine the most suitable antibody pair from three monoclonal antibodies—designated Ab₁, Ab₂, and Ab₃—raised against the same antigen.

A checkerboard titration of Ab₁ against purified antigen is shown in Table 2. Plates were coated with twofold serially diluted antigen starting at 1 µg/mL or 100 ng per well (rows A–H). Ab₁ was added to the wells in Column 1 at 1 µg/mL or 100 ng per well. Ab₁ was twofold serially diluted and added to the remaining columns across the plate (Columns 2–10). Similar checkerboard titrations of Ab₂ and Ab₃ were performed. For all three monoclonal antibodies, the optimal working concentration was 0.5 µg/mL.

Next, these were paired in sandwich ELISAs to determine the best antibody pair to use. To determine the best antibody combination, capture antibody and antigen concentrations were kept constant, while the detection antibody was titrated across the plate in the initial assay (Fig. 2A). Similarly, all possible antibody pairs were analyzed by titration of the antigen across the plate (Fig. 2B). Out of the six possible pairwise combinations, the pairing of Ab₃ as capture antibody (cAb₃) and Ab₁ as detection antibody (dAb₁) resulted in the best detection specificity within the assay.

TABLE 2. Checkerboard titration of antigen and antibody by serial dilution

Antigen (ng per well)	Antibody 1 (ng per well)										
	100	50	25	12.5	6.25	3.125	1.563	0.781	0.391	0.195	2'-Ab only
100	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	0.053
50	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	0.079
25	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	0.055
12.5	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	0.103
6.25	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	3.465	0.123
3.125	4.000	4.000	4.000	4.000	4.000	4.000	4.000	3.416	2.970	1.943	0.083
1.5625	2.497	3.031	2.663	2.678	2.639	2.395	2.444	1.793	1.627	1.113	0.058
0.78125	1.168	1.393	1.282	1.103	1.137	1.048	0.964	0.706	0.562	0.485	0.125

FIGURE 2. (A) Detection antibody and (B) antigen titration.

HCP ELISA

The host cell protein (HCP) ELISA is a specialized form of the sandwich ELISA that incorporates polyclonal antibody capable of detecting high- and low-molecular-weight proteins within the complex mixture of a host cell lysate. Development of a HCP ELISA requires that polyclonal antibody is labeled with a reporter of choice. The optimal working concentrations of both unlabeled capture antibody and labeled detection antibody are determined by “scouting,” a process in which antibody reactivity is measured against multiple targets within the mixture. Scouting is performed by diluting the HCP as shown in Figure 3. The antibodies are then titrated against the HCP. Establishment of optimal working concentrations for both capture and detection polyclonal antibodies results in the development of a sandwich ELISA in which sensitive detection of multiple antigens is achieved (Fig. 4).

FIGURE 3. Titration of polyclonal antibody against various dilutions of the HCP mixture.

FIGURE 4. Antibody titration across an optimal predetermined HCP range.

ELISpot Assay

The enzyme-linked immunosorbent spot (ELISpot) assay is based on the sandwich ELISA. Originally developed for the enumeration of antigen-specific antibody-secreting B cells, this assay is now used mainly to enumerate cells within a cell monolayer that produce small cell-signaling molecules. Monoclonal or polyclonal antibodies specific for small cell-signaling molecules are immobilized onto polyvinylidene fluoride or nitrocellulose membranes in microtiter plate wells (Weiss 2012). After blocking, the cells of interest are plated at a predetermined density together with an antigen or mitogen and incubated at 37°C in a humidified CO₂ incubator. Cell-signaling molecules secreted by antigen/mitogen-activated cells are captured by the membrane-immobilized antigen-specific antibody. The filters are washed to remove cells and tissue culture medium, followed by the addition of antigen-specific reporter-labeled antibody. Again, the filters are washed, and an insoluble/precipitating substrate is added. Substrate hydrolysis results in the formation of a colored end product visible as spots on the filter. Each spot represents an individual cell that has produced small cell-signaling molecules. Spots can be counted and their size measured, providing both qualitative and quantitative information (Slota et al. 2011).

RECIPES

Carbonate Buffer for Immunoassays

Reagent	Amount per 1 L of solution
Na ₂ CO ₃	1.59 g
NaHCO ₃	2.93 g
Adjust to pH 9.5 using 1 N NaOH. Store at ambient temperature.	

Phosphate-Buffered Saline (PBS) for Immunoassays (pH 7.4)

Reagent	Amount per 1 L of solution
NaCl	8 g
KCl	0.2 g
Na ₂ HPO ₄	1.44 g
KH ₂ PO ₄	0.24 g
Store at ambient temperature.	

Tris-Buffered Saline (TBS) for Immunoassays

Reagent	Amount per 1 L of solution
NaCl	8 g
KCl	0.2 g
Tris-HCl	3 g
Adjust to pH 7.6 using 1 N NaOH. Store at ambient temperature.	

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